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UTEROTROPHIC RESPONSES AND MODULATION OF UTERINE GENE EXPRESSION INDUCED BY COMBINATIONS OF GENISTEIN AND COUMESTROL IN OVARIECTOMIZED MICE

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ABSTRACT
Genistein and coumestrol are potent dietary phytoestrogens which interact differentially with estrogen receptors and thus mediate comparable estrogenicity. However, data on their combinatory effects *in vivo* are limited. We assessed individual and combined responses on uterine estrogen-sensitive endpoints and uterine gene expression in ovariectomized mice following subcutaneous treatment for 3 consecutive days, 14 d after ovariectomy. Effects on uterine wet weight, luminal epithelial cell height, stromal gland number and uterine ERα, ERβ and PR mRNA expression were determined using qRT-PCR. 17β-estradiol acted as positive control. Coumestrol singly exhibited stronger uterotrophic responses than genistein. However, combinations significantly increased uterine wet weight and luminal epithelial height. Pretreatment with ICI 182,780 revealed ER-dependent activity of the compounds. In contrast to 17β-estradiol, genistein up-regulated ERα while coumestrol up-regulated both ERα and ERβ expression. But, combination resulted in a dose-dependent mixed response, exhibiting marginal up-regulation of ERα at certain dose while significant down-regulation of both ERα and ERβ at other. Thus, endpoint specific and dose-dependent estrogenic responses and differential expression pattern of ERs and down-regulation of PR suggests that the tested compounds may potentially modify sensitivity and physiology of estrogen target organ, which may therefore play significant role in the prevention of endometrial cancer.

INTRODUCTION

Phytoestrogens are diverse group of naturally occurring phenolic, non-steroidal compounds that are natural components of many plant foods (Bacciottini *et al.*, 2007; Thompson *et al.*, 2007) and medicinal plants (Seraphim and Sinha 2010a, 2010b) which exhibit estrogenic activity in vertebrates. Consumption of soy food or phytoestrogens has both beneficial health and adverse effects on development, fertility and the reproductive system (Cederroth *et al.*, 2012). Structurally, phytoestrogens are similar to mammalian endogenous estrogen and thus they can interact with the estrogen receptors alpha and/or beta and trigger the mechanisms of estrogenic action (Davis *et al.*, 1999).

Genistein (Gen), an isoflavone (4', 5, 7-trihydroxyisoflavone) and coumestrol (Coum), a coumestan (7, 12-dihydroxy coumestan) are two potent dietary phytoestrogens found richly in soybean and other legumes (Leuner *et al.*, 2013). They have captured much attention in recent years due to their ability to activate both genomic as well as non-genomic mechanism of actions and their differential interaction with estrogen receptors (ERα and ERβ) (Kuiper *et al.*, 1997) and transactivation (Pike *et al.*, 1999; Mueller *et al.*, 2004), responsible for their comparable estrogenic activity. Gen and Coum compete for binding to the estrogen receptor in immature mouse uterus (Folman and Pope, 1969), but ER-ligand binding assay using rat uterine cytosolic estrogen receptor revealed that relative binding affinities (RBA) of Gen and Coum are much lower than 17β-estradiol (E_2) and Coum has higher RBA than Gen (Branham *et al.*, 2002). Gen and Coum showed a distinct preference for binding to human ERα than for ERβ, but only slight preference for transactivation of ERα compared to ERβ (Mueller *et al.*, 2004). Gen particularly was found to have 20-fold higher binding affinity to ERα than ERβ by solid-phase binding assay (Kuiper *et al.*, 1997, 1998). Determination of the potency of Gen and Coum through *in vitro* studies using recombinant yeast cells containing both human ERα and ERβ found that Coum is more potent than Gen with respect to both receptor subtypes (Bovee *et al.*, 2004). Coum was also found to be 10 times more potent than Gen *in vivo* (Milligan *et al.*, 1998). Although they activate both ERα and ERβ ERE-mediated activities (Li *et al.*, 2013), they are 10^2 to 10^5 times less active than steroidal estrogens (Kuiper *et al.*, 1997, 1998; Schmitt *et al.*, 2001; Morito *et al.*, 2001, 2002).

The low potency of many phytoestrogens suggests that they may have little effect on biological systems at least when studied singly, but in combination they might produce synergistic effects. This concern has led to a number of research initiatives to examine effects of mixture of phyto-and xenoestrogens. Mixture of weak estrogenic chemicals including Gen produced significant additive effects when combined at low concentrations or at concentrations below

no-observed-effect concentration (NOEC) (Silva *et al.*, 2002). Reports are available on synergistic effects of mixture of phytoestrogens like Gen, formononetin, biochanin A and daidzein on anti-atherogenic potential in human umbilical vascular endothelial cells *in vitro* (Andrade *et al.*, 2012), combination of Gen with calcium and vitamin D3 on bone health (Wang *et al.*, 2011), combination of Gen, daidzein and apigenin on steroid hormone secretion (Ohlsson *et al.*, 2010). Other *in vitro* studies also reported the combinatory effects of isoflavones Gen, daidzein, glycetein and formononetin (Willard and Frawley, 1998; Jones *et al.*, 2005; Zhao *et al.*, 2009; Fecteau *et al.*, 2011) and additive effects of mixtures of phytochemicals and synthetic chemicals (van Meeuwen *et al.*, 2007; Katchy *et al.*, 2014). Mixture of isoflavonoids and Coum also regulate estrogen and testosterone production, and modulate ERα and PR expression (Taxvig *et al.*, 2010). Gen and bisphenol A singly and in combination modulated ERα and ERβ expression in hypothalamus-pituitary-gonadal axis and uterus in rats (Yu *et al.*, 2010) and the binary mixture gave intermediate or reduced uterotrophic responses compared with when the components alone (Tinwell and Ashby , 2004).

Estrogen exerts compartment-specific effects on the expression of ERα, ERβ and PR in the adult rodent uterus (Tibbetts *et al.*, 1998; Hiroi *et al.*, 1999) which determines the ultimate effects of estrogens, progesterone (P_4) and other estrogen-like substances in the tissue. Phytoestrogens also have the ability to modify the activity of physiological estrogens by modifying the expression levels of ERs in different estrogen target organs (Penza *et al.*, 2007). Exposure to Gen induces estrogenic responses and decreases estrogen receptor expression (Moller *et al.*, 2009; Xue *et al.*, 2009) and increases estrogen synthesis (Ye *et al.*, 2009). Gen is also a potency-selective ligand for gene expression regulation by ERα and ERβ (Chang *et al.*, 2008). Moreover, the ratio of ERα:ERβ determines the cell sensitivity and biological responses to estrogen and phytoestrogens (Konduri and Schwarz, 2007; Bottner *et al.*, 2014). In view of these facts, we assessed the effects of Gen, Coum and their combination on expression of uterine ER and PR as general response markers for estrogenicity.

Although there are number of reports on uterotrophic effects of Gen and Coum alone *in vivo* in rats (Markaverich *et al.*, 1995; Tinwell *et al.*, 2000; Zhang *et al.*, 2008; Al-Nakkash *et al.*, 2010), there is paucity of literature on uterotrophic effect and expression of uterine estrogen response markers of binary mixture of the two. Moreover, in view of the preferential binding of Gen and Coum to ERα and the report that activation of ERβ may modulate ERα-mediated physiological effects *in vivo* and substances with selective affinity for ERβ are able to antagonize distinct physiological functions (Hertrampf *et al.*, 2008), evaluation of the combinatory uterotrophic responses of these compounds may have interesting result.

The ovariectomized mouse uterotrophic bioassay is the most common short-term *in vivo* assay for (anti) estrogenicity, suitable for screening ERα agonists and antagonists (Ohta *et al.*, 2012) and is highly recommended by the Organization for Economic Co-operation and Development (OECD) (Owens and Koeter, 2003). Therefore, the present study aims at investigating the single and combinatory effects of Gen and Coum in ovariectomized C3H/He mice. We evaluated the effects on uterine wet weight and other highly estrogen-sensitive morphological endpoints like luminal epithelial height and stromal gland number. Influence of antiestrogen was studied to determine whether the estrogenic responses of compounds are ER-dependent or not. In addition, we studied the individual and combined effect on mRNA expression of uterine estrogen response markers like estrogen receptors (ERα and ERβ) and progesterone receptor (PR) using quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Chemicals and test compounds

Estradiol (1,3,5 [10]- Estratriene-3, 17β-diol) Sigma, Germany (used as positive control), genistein (4',5,7-trihydroxyisoflavone, Sigma-Aldrich, China) and coumestrol (7,12-dihydroxy coumestan, Fluka, Sigma-Aldrich, USA) and fulvestrant or faslodex (ICI 182,780) (Sigma-Aldrich, Israel) were purchased from Sigma. The minimum purity of all compounds was 98%. Ketamine (Aneket, Ketamine hydrochloride) and Xylaxine (Xylaxine hydrochloride; Indian Immunologicals Ltd, Hyderabad) were used as anaesthesia. Olive oil (Sigma) was used as vehicle. TRIzol® reagent (Invitrogen, USA; Cat No. 15596-026) was used for isolation of total uterine RNA. It contains phenol (<50%) and guanidine thiocyanate. Thermo Scientific RevertAid First strand cDNA synthesis kit (Fermentas, K-1621) was used for cDNA preparation. Power SYBR Green 2X PCR Master Mix (Applied Biosystems, Warrington, UK; Lot No. 1109302, P/N-4367659) was used for determination of gene expression level.

Preparation of doses of test substances

Genistein and 17β-estradiol were dissolved in ethanol (Merck, Germany) while coumestrol was dissolved in Dimethylsulphoxide (DMSO, Sigma) to prepare 20mM, 100mM and 20mM stocks respectively. Dilution of stock to appropriate doses was done with olive oil for subcutaneous injection. Antiestrogen ICI 182,780 (Fulvestrant) was dissolved

Table 1: Primer pairs used in the study

Genes	Forward primer(Length bp)	Reverse primer(Length bp)	Amplicon size (bp)	Primer Bank ID
Estrogen receptor-α (Esr1)	5'-CTGTCCAGCAGTA ACGAGAAAG-3' (22)	5'-CACAGTAGCGAGTC TCCTTGG-3' (21)	64	145966838b2
Estrogen receptor-β (Esr2)	5'-GTAGAGAGCCGT CACGAATACT-3' (22)	5'-GGTTCTGCATAGA GAAGCGATG-3' (22)	197	46877093b2
Progesterone receptor (Pgr)	5'-CTCCCAGACGGA AAGACAGG-3' (20)	5'-CCTTCCCTATGA GTGGCTTCT-3' (21)	86	112363097b2
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	5'-AGGTCGGTGT GAACGGATTTG-3' (21)	5'-GGGGTCGTTG ATGGCAACA-3' (19)	95	126012538b1

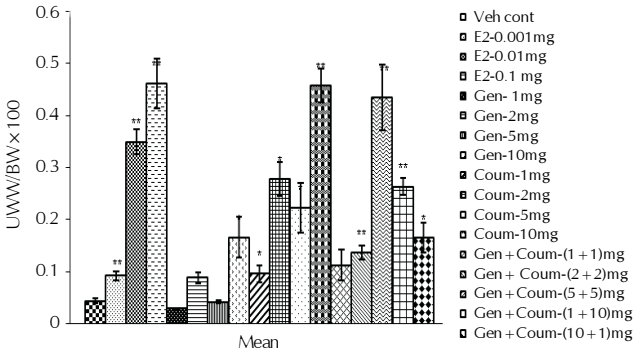

Figure 1: Dose dependent effects of E_2, Gen, Coum and their various combinations on uterine wet weight (UWW/BW × 100) in ovariectomized adult mice. Values represent mean ± SEM, *n* = 4. Error bars indicate SEM. Experimental conditions and treatment procedures are described in Materials and Methods. *Abbreviations*: E_2: 17α-estradiol; Gen: genistein; Coum: coumestrol; Veh cont: vehicle control; UWW: uterine wet weight; BW: body weight. *p < 0.05 and **p < 0.01 indicate significance against vehicle control (William S Cosset's t-test or Student's t-distribution)

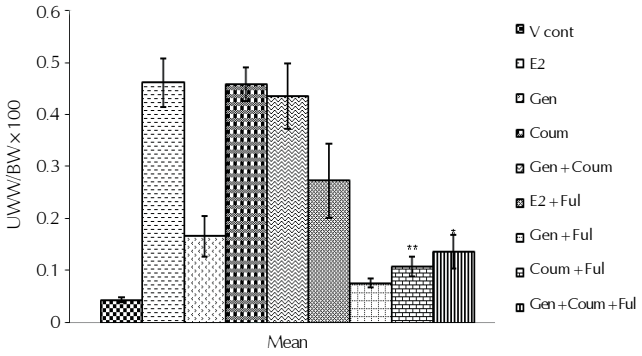

Figure 2: Effect of antiestrogen ICI 182, 780 (1mg/kgBW) on uterine wet weight induced by E_2 (0.1mg/kgBW), Gen (10mg/kgBW), Coum (10mg/kgBW) and their combination (5mg/kgBW each) in ovariectomized adult mice. Values represent mean ± SEM, *n* = 4. *Abbreviations*: E_2: 17α-estradiol; Gen: genistein; Coum: coumestrol; Ful: ICI 182,780; V cont: vehicle control. *p < 0.05 and **p < 0.01 indicate significance against corresponding treatment without Ful (William S Cosset's t-test or Student's t-distribution). Experimental conditions and treatment procedures are described in Materials and Methods

in DMSO to prepare 10mM stock. This stock was then appropriately diluted with olive oil to prepare desired dose.

Animals and treatment

Juvenile female albino mice (C_3H/He strain) weighing 20-25g were obtained from the Departmental Animal House Facility of Gauhati University (Guwahati, Assam, India). The animals were maintained under controlled conditions of temperature (25 ^{0}c ± 2) and illumination (12 h light, 12 h darkness). All the animals had free access to the mixture of commercially available animal diet (containing maize-powder, oilcake, corn-husk and salt) with Vitamin supplement ('Agrimin Forte', Vitamin and mineral supplement for animal) and water. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and according to accepted veterinary medical practice and the Universities Federation for Animal Welfare (UFAW), London handbook (Lane-Petter, 1957). Organization for Economic Co-operation and Development (OECD, 2007) guideline was specifically followed for studying estrogenic activity.

Mice were ovariectomized bilaterally under Ketamine-Xylaxin anaesthesia both at 50mg/kgBW i.m. (Mulder and Mulder, 1979) and randomly allocated to treatment or vehicle groups. After 14 days of endogenous hormonal decline the animals were treated with the test compounds and their combinations through subcutaneous injection in 0.1ml for 3 consecutive days. 17β-estradiol was used as reference. Administration of antiestrogen ICI 182,780 at 1mg/kgBW was done subcutaneously for 3 consecutive days, at least 1 h prior to administration of test compounds (Milligan *et al.*, 1998). Mice were euthanized 24 h after the completion of treatment by cervical dislocation following light ether anaesthesia.

Determination of uterine wet weight

Uterine tissues were dissected out and fats if any were trimmed away rapidly. Uterine weight was measured immediately to avoid desiccation of the tissues, using Sartorius Electronic Balance (Germany). To compensate the effect of body weight on uterine weight, the ratio of uterine wet weight and body weight was calculated for each animal by dividing the uterine weight by the body weight and multiplying by 100 (UWW/BW×100) (Banks *et al.*, 2001).

Determination of uterine luminal epithelial cell height

To determine luminal epithelial cell height, we obtained measurement from H&E stained 5 μm thick longitudinally embedded uterine tissue sections, always from the middle of the uterus and away from the oviduct or cervical segments (microscopic constant = 0.025, Labomed ATC 2000). At least three measurements were taken from 3 random areas from a minimum of four animals (3 areas/tissue/animal or at least 12 measurements per group). We calculated the mean for each animal. These animal means were used to determine the mean ± SEM for each treatment group (Banks *et al.*, 2001).

Determination of stromal gland number

We counted the number of glands from H&E stained 5 μm thick whole uterine cross-sections obtained from the middle region of the uterus. Counting was done in three sections per animal from a minimum of four animals per treatment group (3 tissue sections/animal/group or a minimum of 12 counts per group). Mean for each animal was calculated. These animal means were used to determine the mean ± SEM for each treatment group (Banks *et al.*, 2001).

RNA- extraction, reverse transcription and real-time PCR

The total cytoplasmic RNA was extracted from the uteri using the standard TRIzol® method (Invitrogen, USA). Concentration of total RNA was measured using Biophotometer Plus (Eppendorf) and stored at -20°C. RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific RevertAid First strand cDNA synthesis kit, Fermentas, K-1621) and oligo $(dT)_{18}$ primer were used for first-strand cDNA synthesis. The quantization of cDNA was done using Biophotometer Plus (Eppendorf) and reverse transcription reaction product was stored at -20°C.

Real-time PCR was performed with Power SYBR Green 2X PCR

Master Mix (Applied Biosystems, Warrington, UK) using Step One™ 48-well Real-Time PCR system Thermal Cycling Block (Applied Biosystems, S/N-271002515) with Step One™ software v. 2.1. All reactions were run in triplicates. The 10 μl PCR reaction recipe was pipetted in each well of Fast Optical 48-well Reaction Plate (MicroAmp, Applied Biosystems). The PCR reaction was run at standard ramp speed (~2 h to complete a run). The PCR reactions consisted of a first denaturing cycle at 95°C for 10 min, followed by 40 cycles of 45 s at 95°C, 1 min at 56°C and 1 min at 72°C. Fluorescence was quantified during the 56°C annealing step and the product formation was confirmed by melting curve analysis (60-95°C @ 0.3°C interval). The sequences of primers with Primer Bank ID are shown in table 1.

The comparative C_T ($\Delta\Delta C_T$) method was followed for quantitation of gene expression. Relative mRNA amounts of target genes were calculated after normalization to the endogenous reference gene glyceraldehyde phosphate dehydrogenase (Gapdh), and relative to the negative control (no template control) with the arithmetic formula $RQ = 2^{-\Delta\Delta CT}$ (Winer *et al.*, 1999). Values of Relative Quantity (RQ) were used to prepare graph.

Statistical analysis

The results were expressed as mean ± standard error of mean (x ± SEM). The means of the different treatment groups were analyzed by one-way ANOVA. For comparison of selected means William S Cosset's t-test or Student's t-distribution were used. Significance in most of the cases was assumed both at 5% and 1%, while for gene expressions significance at 0.1% level was also considered.

RESULTS

Uterine wet weight

The result of uterine wet weight expressed as the ratio of wet weight to body weight shows (Fig. 1) that E_2 significantly ($P<0.01$) increased wet weight in a dose-dependent manner. All doses of Coum and highest dose of Gen (10mg/kgBW) also significantly ($P<0.05$ or $P<0.01$) stimulated this endpoint against vehicle control. Effect of Coum at the same doses were also significantly higher compared to Gen ($P<0.05$ or $P<0.01$). Combination of Gen and Coum at 2 mg/kgBW and 5 mg/kgBW each resulted in significant increase in uterine weight ($P<0.01$). Combinations at 1 and 5 mg/kgBW each also exhibited synergistic effect since resultant responses are higher compared to that of individual compounds. Gen at all doses appears to antagonize Coum-induced responses. Interestingly, although the effects of combination of lowest (1mg/kgBW) and highest (10mg/kgBW) doses of Gen and Coum respectively were significant compared to vehicle control, combination comprising highest dose of Coum (10mg/kgBW) resulted in higher effect. One-way analysis of variance (ANOVA) shows that the means of various treatments differ significantly ($P<0.01$).

Influence of antiestrogen on uterine wet weight

To examine whether uterotrophic effects mediated by Gen and Coum in ovariectomized mice are ER-dependent or not, we assessed effect of pretreatment of pure antiestrogen Fulvestrant-ICI 182, 780 (Ful) on increase in uterine wet weight. The result shows (Fig. 2) that Ful significantly reduced uterine wet weight induced by Coum and combination of Gen and Coum, compared to corresponding treatments without Ful ($P<0.01$ and $P<0.05$ respectively).

Uterine luminal epithelial height

Figure 3A shows the result of changes in uterine luminal epithelial cell height measured in micrometer. E_2 (0.1mg/kgBW), Gen (2mg/kgBW), Coum (2mg/kgBW) and combination of Gen and Coum (both at 2mg/kgBW) significantly ($P<0.01$) increased epithelial height compared to vehicle control. Although, the effect of combination of Gen and Coum was significantly higher against Gen alone ($P<0.01$), it was significantly lower against Coum alone ($P<0.05$), indicating antagonistic behaviour of Gen on Coum-induced increase in luminal epithelial cell height. Similar to the effect on uterine wet weight, Coum-induced stimulation of this endpoint was also found to be stronger than Gen. One-way analysis of

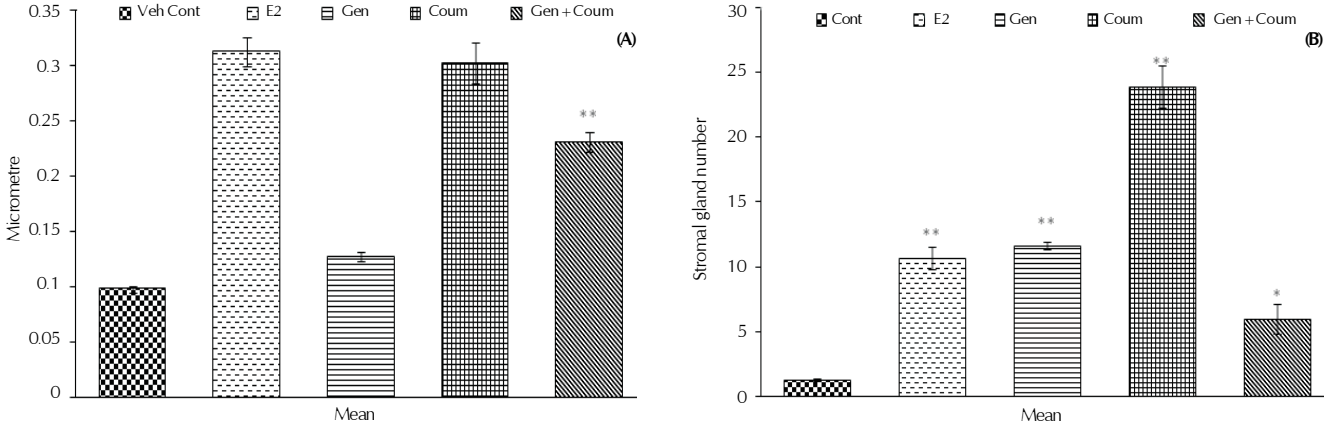

Figure 3: Combined effects of E_2 (0.1mg/kgBW), Gen (2mg/kgBW) and Coum (2mg/kgBW) on the A) uterine luminal epithelial height (μm) and B) uterine stromal gland number in ovariectomized adult mice. Values represent mean ± SEM, $n=4$. Error bars indicate SEM. Experimental conditions and procedure of epithelial height measurement and counting of gland numbers are described in Materials and Methods. *Abbreviations*: E_2: 17β-estradiol; Gen: genistein; Coum: coumestrol; Veh cont: vehicle control. *$p<0.05$ and **$p<0.01$ indicate significance against vehicle control (William S Cosset's t-test or Student's t-distribution).

variance (ANOVA) shows that the means of various treatments differ significantly ($P < 0.01$).

Uterine stromal gland number

As shown in Fig. 3B, E_2, Gen and Coum alone resulted in significant increase in uterine stromal gland number against control ($P < 0.01$). Interestingly, the effect of Coum (2mg/kgBW) was highly significant ($P < 0.01$) compared to both E_2 (0.1mg/kgBW) and Gen (2mg/kgBW) indicating stronger stimulation of stromal glandular proliferation by the former. Combination of Gen and Coum (both at 2mg/kgBW) showed significantly lower effect compared to the individual compounds ($P < 0.01$), indicating potentiating antagonistic behaviour of Gen in presence of Coum. One-way analysis of variance (ANOVA) shows that the means of various treatments differ significantly ($P < 0.01$).

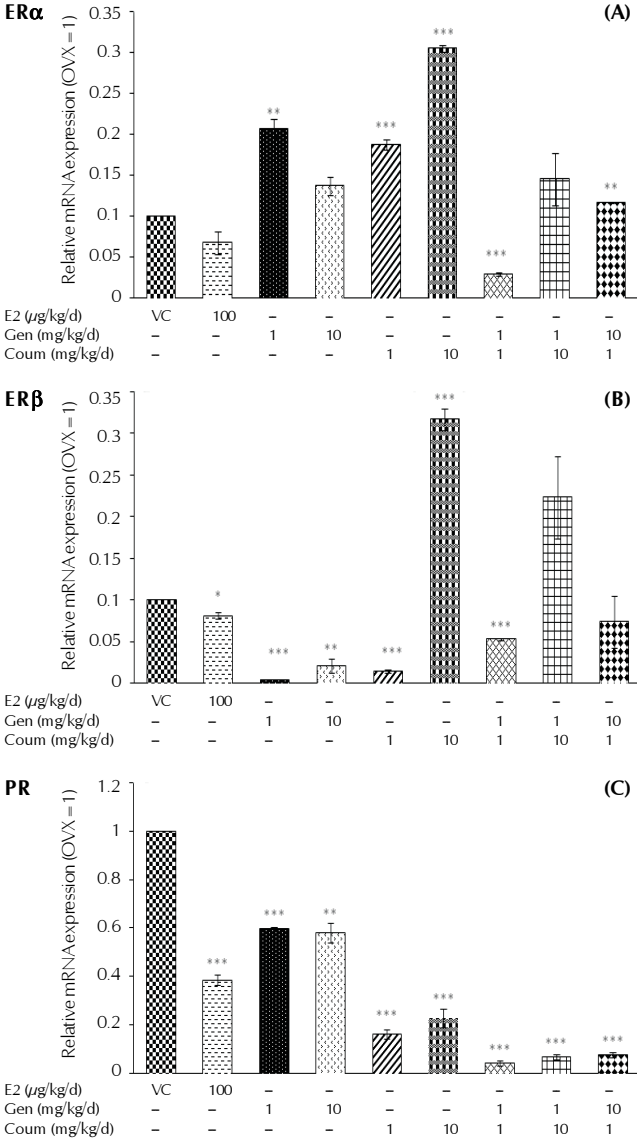

Figure 4: Relative uterine mRNA expression levels of A) **estrogen receptor-α (ERα) B) estrogen receptor-β (ERβ) and C) progesterone receptor (PR) in ovariectomized mice, measured in the respective treatment groups. Error bars indicate SEM (*n* = 3). *Abbreviations*: E_2: 17β-estradiol; Gen: genistein; Coum: coumestrol; VC: vehicle control. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate significance against vehicle control (William S Cosset's t-test or Student's t-distribution). Experimental conditions and procedure of relative quantification of gene expression are described in Materials and Methods**

ERα mRNA expression

As shown in the Fig. 4A, E_2 down-regulated ERα mRNA expression. In contrast, Gen (1mg/kgBW) and Coum (1mg/kgBW and 10mg/kgBW) significantly up-regulated ERα expression individually. Interestingly, however, combination of Gen and Coum (1mg/kgBW each) resulted in down-regulation of ER significantly ($p < 0.01$) and also synergistically. Although reciprocal combination of highest (10mg/kgBW) and lowest (1mg/kgBW) doses of Gen and Coum respectively resulted in up-regulation of ER, significant ($p < 0.01$) effect was obtained in case of combination having higher dose of Coum.

ERβ mRNA expression

In contrast to the ERβ expression, almost all the treatments significantly ($p < 0.05$, 0.01 or 0.001) down-regulated ERβ expression compared to vehicle control (Fig. 4B), except Coum (10mg/kgBW) alone and its combination with Gen. This indicates that Coum at higher doses have the potential to up-regulate ERβ expression. Although, combination of Gen and Coum at (1 + 1) mg/kgBW and (10 + 1) mg/kgBW appear to synergistically up-regulate ERβ expression, the resultant effects were lesser compared to vehicle control. On the other hand, the resultant effect of combination of Gen and Coum at (1 + 10) mg/kgBW shows significant up-regulation of expression compared to vehicle control ($P > 0.05$). In combination, Gen appeared to antagonize Coum-induced up-regulation of ERβ expression.

PR mRNA expression

Similar to the effect on ERβ, all the treatments significantly down-regulated PR expression (Fig. 4C) compared to the vehicle control ($p < 0.01$ or 0.001), although a dose dependent increase in PR expression was observed for E_2. The down-regulating effects of Coum at different doses have been found to be stronger than Gen. However, all combinations of Gen and Coum synergistically down-regulated PR expression, since the resultant responses in all cases are lower than that of the corresponding individual components at the same doses.

DISCUSSION

The data presented in this paper confirmed comparable estrogenicity of Gen and Coum *in vivo*. Although both compounds significantly stimulated all uterine endpoints, Coum has been found to be more potent than Gen in this short-term uterotrophic assay in ovariectomized mice. Thus, our results bear strong resemblances with other *in vitro* and *in vivo* reports (Folman and Pope, 1966; Kuiper *et al.*, 1998; Milligan *et al.*, 1998; Jefferson *et al.*, 2002). Uterotrophic responses of soy isoflavones (Santos *et al.*, 2010) and faint stimulation of uterine wet weight (Diel *et al.*, 2004) and poor induction of Proliferating Cell Nuclear Antigen (PCNA) mRNA and protein expression in the uterine epithelium (Schmidt *et al.*, 2006) by Gen following 3d exposure in ovariectomized rats provide the clues in favour of weaker uterotrophic response

of Gen observed in the present study. However, at higher doses (25, 50, or 100mg/kgBW), Gen dose-dependently increased uterine wet weight and epithelial height (Diel *et al.*, 2001) and increased uterine weight and gland number (at 125 or 250mg/kg) (Carbonel *et al.*, 2011) in rat.

Gen is estrogen receptor-β agonist while Coum has higher binding affinity with estrogen receptor-α (Barkhem *et al.*, 1998; Kuiper et al., 1998; Lee et al., 2004; Turner et al., 2007). The lower uterine weight obtained for ERβ agonist like Diarylpropiol nitrile (DPN), compared to ERα agonists E_2 and propyl pyrazole triol (PPT) in ovariectomized rats (Blesson *et al.*, 2012) also strongly supports weaker uterotrophic response obtained for ERβ agonist Gen compared to ERα agonists E_2 and Coum. Although Gen exhibits preferential binding to ERβ in receptor binding assay (Kuiper *et al.*, 1997), it induces transcription of ERα mRNA (Cotroneo *et al.*, 2001) or induces estrogen responsive genes via ERα-mediated mechanisms in the immature rat uterus (Lee *et al.*, 2004). Therefore, estrogenic activity of Gen in the uterus is a factor of distribution of the two ER-subtypes in the uterus and its interaction with the ERs.

In the present study, Gen in combination with Coum mediated dose-dependent mixed agonistic/antagonistic effects with respect to increase in uterine wet weight, with potentiating synergism at 1 or 5 mg/kgBW and antagonism at 2mg/kgBW. Similarly, combination significantly stimulated estrogen-sensitive morphological endpoints in the uterus *viz.*, uterine luminal epithelial height and stromal gland number. However, the resultant responses of combination in all the uterine morphologic endpoints obtained in our study were lower than those of Coum alone. This is indicative of antagonistic behaviour of Gen in presence of Coum. Similarly, binary mixture of Gen and bisphenol A also gave an intermediate or reduced uterotrophic response compared to the components alone (Tinwell and Ashby , 2004). This nature of combinatory response of estrogenically weak substances is of considerable concern given the exposure of human and animals to such environmental chemicals. The antagonistic effect of Gen may be explained in terms of its selective higher binding affinity for ERβ (Kuiper *et al.*, 1998) and induction of ERα or its bioavailability and competition with Coum for binding to the ERs. The report that activation of ERβ may modulate ERα-mediated physiological effects in uterus (Weihua *et al.*, 2000) and substances with selective affinity for ERα are able to antagonize distinct physiological functions (Hertrampf *et al.*, 2008) also provide important clues to explain the antagonistic behaviour of Gen observed in the present study.

Reduction of uterine wet weight following pre-treatment with antiestrogen ICI 182,780 clearly indicated that Gen- and Coum-induced increase in uterine wet weight is ER-dependent, since ICI 182,780 acts as an estrogen receptor down-regulator (Robertson, 2001) and also suppress the expression of estrogen-dependent genes (Howell *et al.*, 2000).

In the present investigation, E_2 exposure for three consecutive days down-regulated ERα, ERβ and PR mRNA expression in ovariectomized mice. Other reports on down-regulation of ERα, ERβ and PR mRNA levels by E_2 (4μg/kgBW) following consecutive 3 days treatment in ovariectomized rat (Manni *et al.*, 1981; Diel *et al.*, 2004; Diel *et al.*, 2006) strongly supports our findings. In contrast, Gen alone up-regulated ERα while down-regulated ERβ and Coum alone significantly up-regulated both ERα and ERβ expression. However, a dose-dependent mixed response was obtained for combination, down-regulation of both ERα and ERβ at 1 mg/kg, while marginal up-regulation of ERα, but not ERβ for combination of low and high dose (1mg/kg and 10mg/kg). Interestingly, all combinations also exhibited negative synergistic effect with regard to ERα expression since the resultant responses for combinations were lower than that of individual components alone. Significant up-regulation of both estrogen receptors by Coum probably offers valuable evidence to support comparable estrogenicity of Gen and Coum. In addition, considering the association and importance of ERβ in ovarian and endometrial cancer (Chakravarty *et al.*, 2008; Haring *et al.*, 2012) and colon cancer (Bielecki *et al.*, 2011), Coum may emerge as a potential therapeutic agent to prevent such cancers.

Up-regulation of ERα in ovariectomized rats (Diel *et al.*, 2006) and down-regulation in intact post-weaning rats (Zin *et al.*, 2013) by Gen (10 or 100mg/kg) clearly indicates that Gen-induced ERα expression varies in ovariectomized and intact animals and may possibly depends on the estrogen status of the animals. Although Gen exhibits preferential binding to ERβ in receptor binding assay (Kuiper *et al.*, 1997), it induces transcription of ERα mRNA (Cotroneo *et al.*, 2001) or induces estrogen responsive genes via ERα-mediated mechanisms in the immature rat uterus (Lee *et al.*, 2004). Thus, the molecular mechanisms involved in the uterine activity of Gen are very complex and may be distinct from those of endogenous estrogens. Comparison of the activity of Gen with E_2 clearly demonstrates that treatment of animals with Gen mimics to a considerable degree typical morphological and molecular properties of estrogens in the uterine tissue. However, Gen has very limited ability to induce tissue proliferation.

Regulation of receptors by their cognate ligand (autoregulation or homologous regulation) is a common feature of steroid and other members of the nuclear receptor superfamily (Burnstein and Cidlowski,1993). As a rule, sex steroid receptors are down-regulated by cognate ligand (negative autoregulation). This probably offers the important clue to presume that E_2 may preferably activate ERα in the uterus and thus leads to its down-regulation. Gen and Coum perhaps failed to activate ERα as strongly as E_2 with the result that ERα expression increased in the uterus.

Since progesterone receptor (PR) is an estrogen-responsive gene in the uterus, we have considered PR expression as an indicator of ER-mediated transcription and to evaluate the agonistic/antagonistic effects of Gen, Coum and their combination. We demonstrated significant down-regulation of PR mRNA expression for E_2, Gen, Coum and the combinations of the latter two. The down-regulating effects of Coum and its combination with Gen appeared to be stronger than the later. All combinations synergistically down-regulated PR expression, since the resultant responses in all cases were lower than that of the corresponding individual components at the same doses. Decrease in PR mRNA despite continued exposure to E_2 (Kraus and Katzenellenbogen,1993) and down-regulation of PR by E_2 and Gen in both intact and ovariectomized rat after 3 d exposure (Diel *et al.*, 2004; Diel *et*

al., 2006) supports our findings. It has been speculated that large doses of estrogen (25 or 50 μg) significantly decrease estrogen receptor content in the rat uterus, which in turn inhibits synthesis of PR (Manni *et al.*, 1981).

Finally, the present study offers evidence in favour of differential estrogenicity of Gen and Coum, and most importantly shows that combinatory effects of estrogenically weak compounds may not always be additive, as expected. In addition, we also demonstrated that potent dietary phytoestrogens singly and in combination can modulate estrogenic responses in a dose and endpoint-specific manner and may also regulate estrogen receptor expression in a manner different from endogenous estrogen. We, therefore, conclude that acting via classical pathway non-steroidal plant-derived natural compounds like Gen and Coum do have the potential to modulate ER-mediated effects and may modify sensitivity and physiology of estrogen target organ through modulation of estrogen receptors, which may have important implications with regard to the incidence of endometrial cancer.

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